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Superantigen Recognition by γδ T Cells: SEA Recognition Site for Human Vγ2 T Cell Receptors

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Summary

Human γδ T cells expressing the Vγ2Vδ2 antigen receptors recognize nonpeptide prenyl pyrophosphate and alkylamine antigens. We find that they also recognize staphylococcal enterotoxin A superantigens in a manner distinct from the recognition of nonpeptide antigens. Using chimeric and mutant toxins, SEA amino acid residues 20–27 were shown to be required for γδ TCR recognition of SEA. Residues at 200–207 that are critical for specific αβ TCR recognition of SEA do not affect γδ TCR recognition. SEA residues 20–27 are located in an area contiguous with the binding site of V β chains. This study defines a superantigen recognition site for a γδ T cell receptor and demonstrates the differences between Vγ2Vδ2$^+$ T cell recognition of superantigens and nonpeptide antigens.

Introduction

T cells can be divided into mutually exclusive subsets by their expression of αβ or γδ T cell antigen receptors (TCRs). γδ T cells express distinct TCRs (Porcelli et al., 1991) and are enriched at epithelial surfaces, where they may help to maintain epithelial integrity (Janeway, 1988; Boismenu and Havran, 1994). Emerging evidence from clinical studies on human infections (reviewed in Morita et al., 2000) and studies on mice with targeted disruption of γδ T cell receptors suggests an important role for γδ T cells in adaptive immunity to infections (Ladel et al., 1995) and in innate immunity (Janeway et al., 1988; Boismenu and Havran, 1997). The major subset of human γδ T cells has been shown to expand greatly in response to a number of bacterial and parasitic infections (reviewed in Morita et al., 2000). γδ T cells in this subset express Vγ2Vδ2 T cell antigen receptors and recognize isopentenyl pyrophosphate and related prenyl pyrophosphate compounds and alkylamine compounds in a TCR-dependent manner (Constant et al., 1994; Bukowski et al., 1995, 1998, 1999; Tanaka et al., 1995). This recognition has been said to be "superantigen-like" because responding Vγ2Vδ2$^+$ T cells have diverse junctions in their TCR. Since isopentenyl pyrophosphate and related molecules are required for the biosynthesis of essential compounds by both prokaryotic bacteria as well as eukaryotic protozoa, the Vγ2Vδ2$^+$ T cell subset can respond to infections by a number of different pathogens. Prenyl pyrophosphate antigens can be presented through a direct extracellular pathway that requires neither antigen processing nor known antigen-presenting molecules (Lang et al., 1995; Morita et al., 1995). Thus, γδ T cell recognition of nonpeptide prenyl pyrophosphates may complement αβ T cell recognition of conventional protein antigens and lipid antigens.

In contrast to γδ T cells, most αβ T cells specifically recognize foreign peptides presented by MHC class I or class II molecules or lipid/glycolipids presented by CD1. αβ T cells are also stimulated by another class of antigens, superantigens. Superantigens are either exogenous toxins produced by staphylococci, streptococci, and other bacteria, or endogenous proteins encoded by murine mammary tumor viruses, rabies viruses, or other viruses (Scherer et al., 1993; Huber et al., 1996). Superantigens are a major source of food poisoning and are etiologic agents of staphylococcal and streptococcal toxic shock syndrome. Superantigens have binding sites for nonpolymorphic regions of MHC class II molecules outside of the antigen binding groove (Kozono et al., 1995) and for the Vβ domain of the αβ T cell receptor (Li et al., 1998a). Whereas most murine and human αβ T cells respond to one or more superantigens, no murine γδ T cells have been identified that respond to exogenous or endogenous superantigens (O'Brien et al., 1989; Chandler et al., 1995).

In addition to recognizing nonpeptide antigens (Morita et al., 1996), it has been suggested that the major human Vγ2Vδ2$^+$ T cell subset recognizes the staphylococcal enterotoxin A (SEA) superantigen because Vγ2Vδ2$^+$ T cells killed B cell lines pulsed with SEA (Rust et al., 1990). Unlike its effects on αβ T cells, however, SEA generally did not stimulate the proliferation of Vγ2Vδ2$^+$ T cells (Rust et al., 1990). Subsequently, it was reported that most of the γδ T cell clones were killing SEA-pulsed targets through antibody-dependent cellular cytotoxicity due to antibodies to SEA that were present in human serum used in the assay (Rust et al., 1993a). However, a few γδ T cell clones continued to kill SEA-pulsed targets in the absence of antibodies to SEA and to prolifer-

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ate in response to staphylococcal superantigens (Rust et al., 1993b; Ramesh et al., 1995; Stinissen et al., 1995a, 1995b). Reactivity to SEA correlated with the expression of the Vγ2 chain in TCR transfection studies (Loh et al., 1994).

In this study, we report that $V\gamma2^+$ T cells can proliferate to the superantigen, SEA, and that recognition of SEA is distinct from the recognition of prenyl pyrophosphate antigens. Using SEA mutants, we have mapped critical amino acids on SEA that determine γδ T cell recognition to an area that is structurally contiguous with the known binding site on SEA for the Vβ domain. These results define a superantigen binding site for a γδ T cell receptor and clearly demonstrate the differences between the recognition by $V\gamma2V\delta2^+$ T cells of a classical superantigen and of nonpeptide prenyl pyrophosphate and alkylamine antigens.

Results

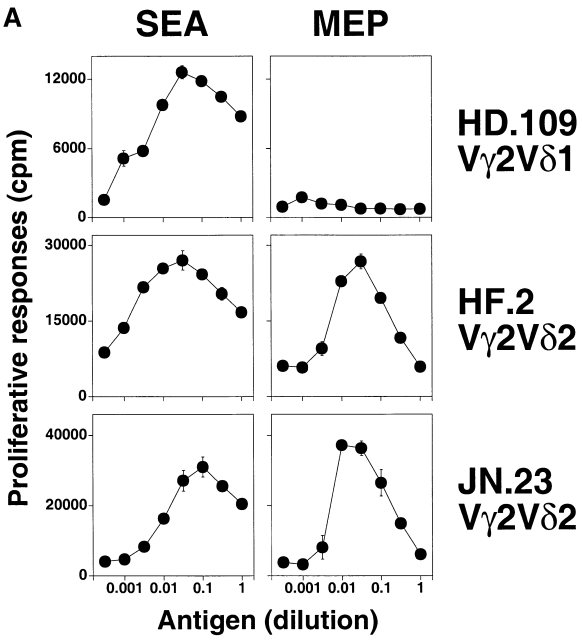
γδ T Cell Recognition of SEA Is Distinct from the Recognition of Prenyl Pyrophosphates

In contrast to an earlier study (Rust et al., 1990), γδ T cell clones expressing Vγ2 TCR were able to proliferate when stimulated by SEA (Figure 1A). Unlike protein antigens, superantigens do not require internalization or intracellular processing and thus can be presented by fixed antigen-presenting cells (APCs) similar to the presentation of prenyl pyrophosphate antigens to γδ T cells (Lang et al., 1995; Morita et al., 1995). Consistent with this fact, the SEA superantigen was presented by fixed cells (Figure 1A). Indeed, for some clones, glutaraldehyde fixation of APCs was required for presentation of SEA (see Experimental Procedures). SEA reactivity was noted with γδ T cells expressing the Vγ2 domain paired with either Vδ2 or Vδ1 whereas reactivity to the isopentenyl pyrophosphate analog, monoethyl phosphate (MEP), required expression of Vγ2 paired with Vδ2 (Figure 1A; Tanaka et al., 1994).

Many γδ T cells respond to prenyl pyrophosphates even in the absence of professional APCs because T cells probably present the nonpeptide antigens to each other (Lang et al., 1995; Morita et al., 1995). Similarly, γδ T cell recognition of alkylamines also does not require professional APCs (Bukowski et al., 1999). Recognition of SEA by γδ T cells, however, required the presence of professional APCs despite the presence of MHC class II on the surfaces of the T cell clones (Figure 1B; Morita et al., 1995). A similar requirement for APC was noted for the response of an αβ T cell line to SED (data not shown). This requirement for APC may reflect the need for additional costimulatory interactions not provided by T cell presentation. Thus, γδ T cell recognition of SEA exhibited fundamental differences when compared with γδ T cell recognition of MEP because γδ T cells expressing Vγ2Vδ1 as well as Vγ2Vδ2 TCRs could respond to SEA and "professional" APCs were required for presentation.

Comparison of αβ and γδ T Cell Responses to SEA

αβ T cell clones were more sensitive to SEA than γδ T cell clones, with half maximal proliferation of αβ T cells being noted at SEA concentrations of 1×10^{-7} to 1×10^{-3} μg/ml as opposed to γδ T cell clones that required



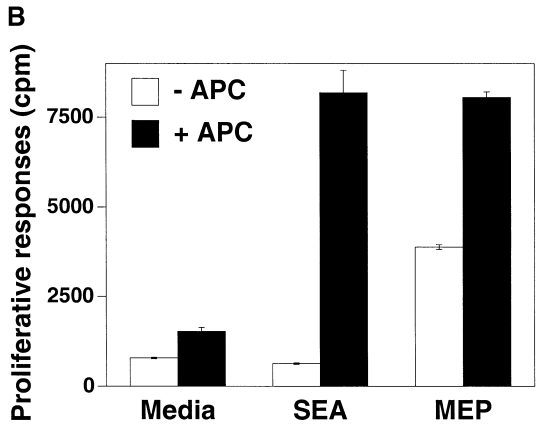


Figure 1. γδ T Cell Clones Bearing Vγ2 TCR Respond to SEA

(A) Response of human $V\gamma2^+$ T cells to the staphylococcal enterotoxin A. EBV-transformed B cells (CP.EBV line) were glutaraldehyde fixed and used as antigen-presenting cells for γδ T cells in the presence of SEA (left panels), MEP (right panels), or PHA (data not shown). rIL-2 was added at 0.03 nM. SEA, MEP, and PHA were added to the cultures at serial one-half log dilutions starting at 3.16 μg/ml for SEA, a 1/100 concentration for MEP, and a 1/100 concentration for PHA. On day 1, each culture was pulsed with ^{3}H-thymidine and harvested 18 hr later. Mean and SEM of triplicate cultures are shown. Note that the $V\gamma2V\delta2^+$ T cell clones, HF.2 (middle) and JN.23 (bottom), responded to SEA and MEP, whereas the $V\gamma2V\delta1^+$ clone, HD.109 (top), only responded to SEA.

(B) γδ T cell recognition of SEA but not MEP requires APCs. The DG.SF68 clone was cultured with SEA (1 μg/ml) or MEP (1/1000 concentration) and DG.EBV B cells with rIL-2 as in (A). Note that the MEP response did not require APCs, whereas the SEA response required APCs.

SEA concentrations of 3.2×10^{-3} to 1×10^{-1} μg/ml (Figure 2). The SEA concentrations required to stimulate γδ T cells are comparable to the SEB concentrations required to stimulate weakly reactive αβ T cells (Surman et al., 1994). Thus, γδ T cells required higher concentrations of SEA for stimulation compared to αβ T cells.

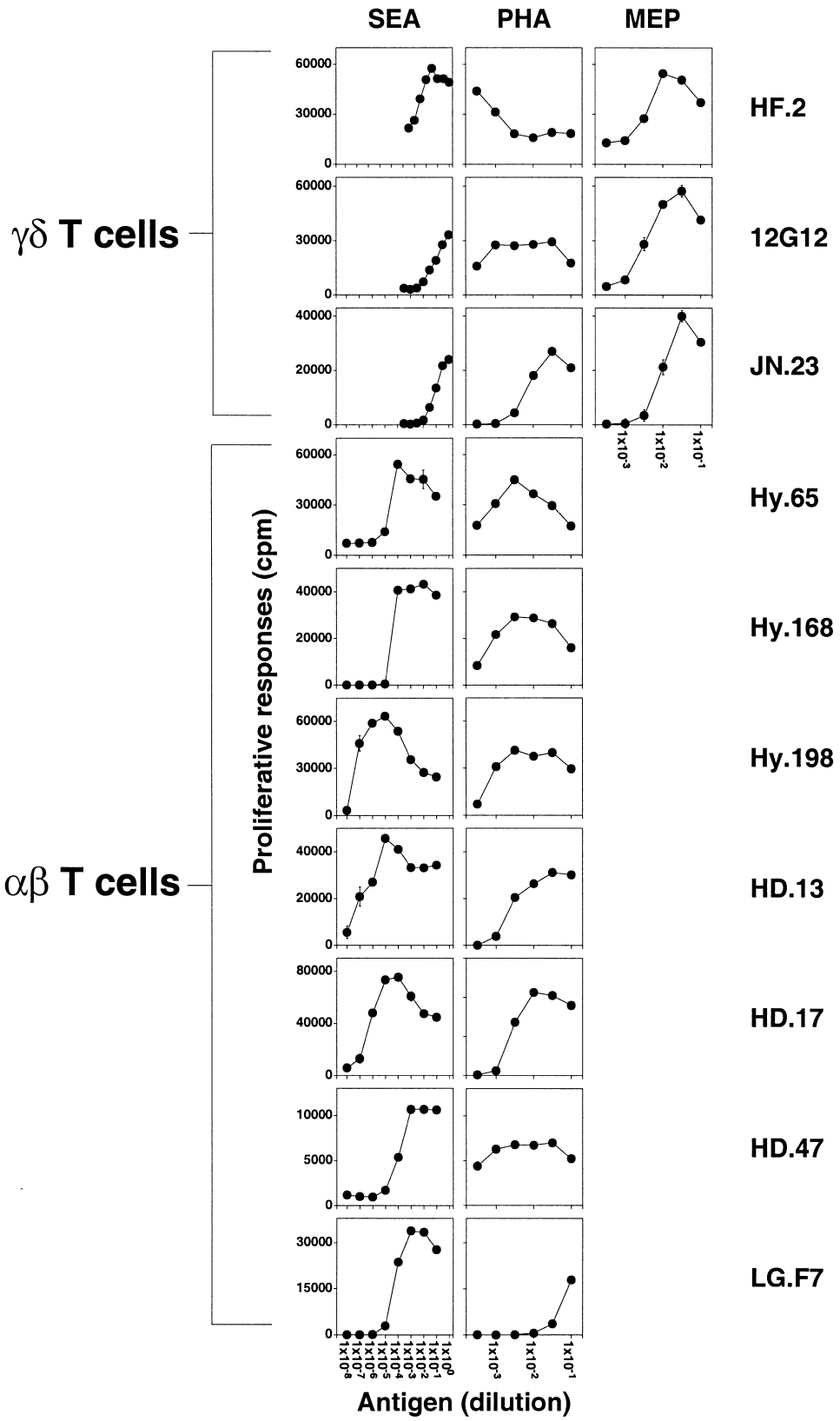


Figure 2. Comparison of γδ and αβ T Cell Responses to SEA

Culture conditions were as in Figure 1A, except rIL-2 was omitted and DG.EBV cells were used. SEA, MEP, and PHA were added at serial one-half or one log dilutions of solutions of SEA (1 μg/ml), MEP (1/100 concentration), and PHA (1/100 concentration). The Hy.65, Hy.168, and Hy.198 αβ T cell clones respond only to SEA and not to SEE, whereas the HD.13, HD.17, HD.47, and LG.F7 αβ T cell clones respond to both.

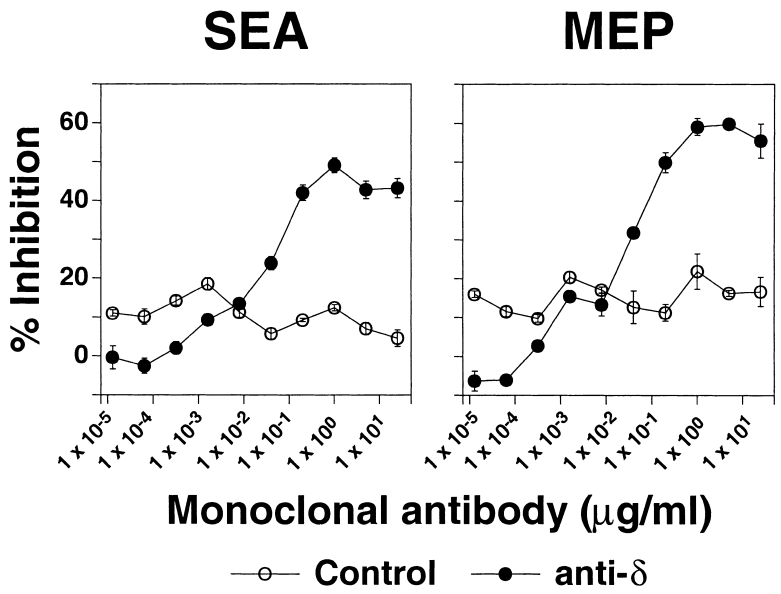


Figure 3. γδ Recognition of SEA Is Mediated through the γδ TCR

Recognition of SEA and MEP were blocked by mAbs to the γδ TCR. Fixed DG.EBV B cells were used to present SEA (1 μg/ml) or MEP (1/3160 concentration) to the γδ T cell clone, HF.2, without rIL-2 as in Figure 1A. Either the control mAb, P3, or the anti-γδ TCR mAb, anti-TCRδ1, was added to the culture at setup. Data are shown as % inhibition [100 − (experimental ^{3}H-thymidine incorporation/control ^{3}H-thymidine incorporation) × 100].

SEA Responses Can Be Blocked by mAbs to the γδ T Cell Receptor, and Responsiveness Correlates with Vγ2 T Cell Receptor Expression

To establish that γδ T cells recognized SEA through the γδ TCR, monoclonal antibodies (mAbs) to the γδ TCR were used to inhibit SEA recognition by γδ T cells. The anti-Cδ mAb but not a control mAb inhibited the recognition of SEA by 49%. This same anti-Cδ mAb inhibited the recognition of the nonpeptide antigen, MEP, by 60% (Figure 3) although responses to the mitogen, PHA, or to IL-2 were not inhibited (data not shown).

Responsiveness to SEA varied between different Vγ2Vδ2$^+$ T cell clones. Thus, five out of seven CD4$^-$8$^-$/CD8$^+$ and two out of two CD4$^+$ γδ T cell clones responded to SEA (Figure 1; data not shown), whereas all of these Vγ2Vδ2$^+$ clones responded to MEP. This variability of responsiveness between different Vγ2Vδ2$^+$ T cell clones may reflect variable expression of inhibitory NK cell receptors (Carena et al., 1997; Halary et al., 1997; Poccia et al., 1997). Unlike previous reports for SEA-induced cytotoxicity (Rust et al., 1993a), there was no correlation between SEA reactivity and the expression of CD16 immunoglobulin receptors (data not shown).

Confirming a previous study with γδ TCR transfectants (Loh et al., 1994), SEA responsiveness correlated with the expression of the Vγ2 TCR chain. Four of five Vγ2Vδ1$^+$ T cell clones responded to SEA whereas none of five Vγ1Vδ1$^+$ T cell clones did so (data not shown). Thus, reactivity to SEA could be specifically blocked by mAbs to the γδ TCR and correlated with Vγ2 expression. These data strongly support the hypothesis that recognition of SEA is through interaction with the Vγ2 domain of the γδ TCR.

Recognition of SEA by γδ T Cells Requires MHC Class II Expression on the APCs and MHC Class II Binding Sites on SEA

Superantigen responses by αβ T cells generally require MHC class II molecules although these responses are not MHC restricted because most MHC class II alleles function as presenting molecules. Three approaches were taken to determine if the recognition of SEA by γδ T cells had a similar requirement for MHC class II molecules. First, the mutant cell line, 721.174, that lacks MHC class II expression due to chromosomal deletions, did not present SEA to the γδ T cell clones, JN.23 and HD.108, whereas the MHC class II–expressing parent line, 721, and mutant MHC class I–deficient line, 721.221, both efficiently presented SEA (Figure 4A, left panels). Consistent with our earlier study (Morita et al., 1995), all three cell lines presented the nonpeptide antigen, MEP, to the JN.23 and HD.108 T cell clones (Figure 4A, right panels). Moreover, in the presence of SEA, Vγ2Vδ2$^+$ T cells lysed the unfixed, MHC class II$^+$ target, Raji, but not the mutant unfixed MHC class II$^-$ target, RJ-2.25 (Morita et al., 1995). Thus, surface expression of MHC class II molecules on the presenting cells was required for SEA recognition by Vγ2Vδ2$^+$ T cells.

Second, mAbs to MHC class II blocked the proliferative response of γδ T cells to SEA to background levels but did not block the response to MEP or to PHA (Figures 4B and 4C; data not shown). mAbs to MHC class I or CD1 molecules had no effect (Figure 4B), whereas mAbs to LFA-1 or γδ TCR inhibited the γδ T cell response to SEA (Figure 4C).

Third, mutation of SEA disrupting either the low-affinity (SEA mutant SEA-F47S.L48S.Y92A) or the high-affinity (SEA mutant SEA-H225A) binding site for MHC class II (Hudson et al., 1995; Kozono et al., 1995) abolished the ability of SEA to stimulate γδ T cells (Figure 4D). Thus, SEA recognition by γδ T cells requires both the presence of MHC class II on the APC surface as well as the low-affinity and the high-affinity binding sites on SEA for MHC class II molecules.

Specific Recognition of SEA by Vγ2$^+$ T Cells

Human Vγ2$^+$ T cells specifically recognized only SEA. Despite the similarity of amino acid sequence between SEA and SEE (83% identity), SEE was at least 1000-fold less active than SEA at stimulating Vγ2$^+$ T cell clones

(Figure 5). Other enterotoxins from *Staphylococcus aureus* (SEB, SEC1, SEC2, SEC3, SED, SEE, or TSST1), *Streptococcus pyogenes* (SPE-A, SPE-B, and SPE-C), or *Mycoplasma arthritidis* (*Mycoplasma arthritidis* mitogen or MAM) did not stimulate Vγ2$^+$ T cell clones (Figure 5; data not shown).

Specific Recognition of SEA by Vγ2$^+$ T Cells Is Dependent on the Amino-Terminal Region of SEA and Is Distinct from Specific Recognition of SEA by Vβ5.2$^+$ αβ T Cells

The murine TCR Vβ8 chain binds to the cleft between the two domains of staphylococcal enterotoxin, SEC2/3 (Fields et al., 1996) and SEB (Li et al., 1998b). To determine if similar residues in SEA are involved in recognition by Vγ2$^+$ T cells, chimeric enterotoxins between SEA and the closely related SEE enterotoxin (83% identity) and mutant SEA enterotoxins were tested for their ability to stimulate Vγ2$^+$ T cell clones (clone HF.2 is shown here) and control Vβ5.2$^+$ T cell clones (Hy.168 is shown here). Chimeric enterotoxins could be used because Vγ2$^+$ T cells and Vβ5.2$^+$ T cells recognized SEA but not SEE enterotoxins (Figures 5 and 6). These chimeric and mutant enterotoxins bind MHC class II molecules with affinities comparable to wild-type (wt) SEA and SEE (SEA has a higher affinity for MHC class II than SEE, varying between 5- to 50-fold higher depending on the MHC class II allele (Herman et al., 1990; Chintagumpala et al., 1991).

Vγ2$^+$ T cell recognition of SEA requires the amino-terminal one-third of SEA (Figure 6A, top left panel, enterotoxin SEA-SEE [86–233]). For full activity, the amino-terminal two-thirds of SEA are required. This difference between the two types of chimeras may reflect the higher affinity for MHC class II (comparable to wt SEA) of chimeras with the amino-terminal two-thirds of SEA (Mollick et al., 1993). Thus, both the SEA-SEE (157–233) enterotoxin and the SEA-SEE (143–233) enterotoxin had full activity (Figure 6A, top left and bottom left panels). Conversely, chimeric SEA enterotoxins lacking this amino-terminal region were not active (for example SEE-SEA [86–233] top left panel, or SEE-SEA [71–233], SEE-SEA [123–233], and SEE-SEA [143–233], bottom left panel). Further confirming the importance of the amino-terminal region of SEA in determining γδ T cell recognition, chimeric enterotoxins with SEE carboxy-terminal regions or mutant SEA toxins with alanine mutations in the carboxy-terminal region (residues 200–207) had normal biological activity for Vγ2$^+$T cells (Figures 6B, top left panel, SEA-SEE [200–207], and 6C, top left panel, SEA-ala [200–207]). This carboxy-terminal region is critical in determining reactivity and specificity for SEA- and SEE-reactive αβ T cells (Hudson et al., 1993; Mollick et al., 1993; Lamphear et al., 1996; see below) and contains an area of contact between SEC2/3 and the murine Vβ8 (mVβ8) chain (Fields et al., 1996).

Amino-terminal residues 20–27 played an important role in determining Vγ2$^+$ T cell recognition of SEA. This area contains four nonconserved residues between SEA and SEE. Changing residues 20 (Gly20Arg) and 21 (Thr21Asp) decreased enterotoxin bioactivity by 10-fold (for example SEA-SEE [20–21/200–207], SEA-SEE [20–27/200–207], and SEA-SEE [20–27]) (Figure 6B, top left panel). Changing only residues 24 (Gly24Ser) and 27 (Lys27Arg) enhanced biological activity 10-fold (SEA-SEE [24–27/200–207]).

Further underscoring the importance of residues 20–27, mutation of the conserved Arg25 residue to alanine abolished SEA biological activity for Vγ2$^+$ T cells (Figure 6B, bottom left panel, SEA-N25A). Also, mutating nonconserved residues to alanine in the 20–24 region diminished SEA activity by 100-fold (Figure 6C, top left panel, SEA-ala [20–24] with G20A, T21A, and G24A mutations). Finally, a second point mutation in residue 64, at the base of a protruding disulfide loop (Schad et al., 1995), enhanced Vγ2$^+$ T cell recognition by 10-fold (Figure 6B, bottom left panel, SEA-Y64A). Mutation of the corresponding residues of SEB also affects αβ T cell recognition (Kappler et al., 1992). These changes in enterotoxin biological activity are readily explained by examining the structure of the SEC2/3-mVβ8 complex. The adjacent residue to Tyr64 in SEC2/3 contacts the Vβ chain and residues 20–27 in SEC2/3 have multiple contacts with the Vβ chain (Figure 7A). Thus, the recognition of SEA by Vγ2$^+$ T cells is greatly affected by changes in residues 20–27 and 64, regions that have been also shown to be important in the recognition of SEB and SEC2/3 by αβ T cells.

In contrast to SEA recognition by Vγ2$^+$ T cells, SEA recognition by Vβ5.2$^+$ T cells is strongly dependent on SEA residues in the carboxy-terminal region. Chimeric enterotoxins at either the amino or carboxy-terminal regions were generally much less active than wt SEA (Figure 6A, top, middle, and bottom right panels). For example, chimeric enterotoxins with the amino-terminal two-thirds of SEA had no change in activity for Vγ2$^+$ T cells but were 10,000- to 100,000-fold less active than wt SEA for Vβ5.2$^+$ T cells (Figure 6A, top panels, SEA-SEE [157–233], and bottom panels, SEA-SEE [143–233]). The carboxy-terminal residues, 200–207, also play a prominent role in determining SEA reactivity. Thus, a chimeric SEE enterotoxin containing only residues 200–207 of SEA had strong biological activity (only 100-fold less than wt SEA) (Figure 6A, middle right panel, SEE-SEA [200–207]). Conversely, replacement of residues 200–207 of SEA with SEE, reduced activity for Vβ5.2$^+$ T cells by 10,000-fold (Figure 6B, top right panel, SEA-SEE [200–207]).

Similar to Vγ2$^+$T cells, the amino-terminal region also played a role in determining SEA recognition by Vβ5.2$^+$T cells. For example, the SEA-SEE (20–27) chimera is 100-fold less active than wt SEA. Mutation of the conserved Arg25 residue abolished bioactivity (Figure 6B, bottom panel, SEA-N25A). In conclusion, specific recognition of SEA by Vγ2$^+$T cells is dependent on residues in the amino-terminal region corresponding to known contact residues on SEB and SEC3 enterotoxins for the mVβ8 chain. Unlike Vβ5.2$^+$T cells and other SEA- or SEE-specific αβ T cells (Figure 6; data not shown), specific recognition of SEA by Vγ2$^+$ T cells appears to be independent of nonconserved residues in the carboxy-terminal region of SEA.

Discussion

In this study, we define the recognition site on SEA for γδ T cells expressing Vγ2$^+$TCR. We find that superantigen

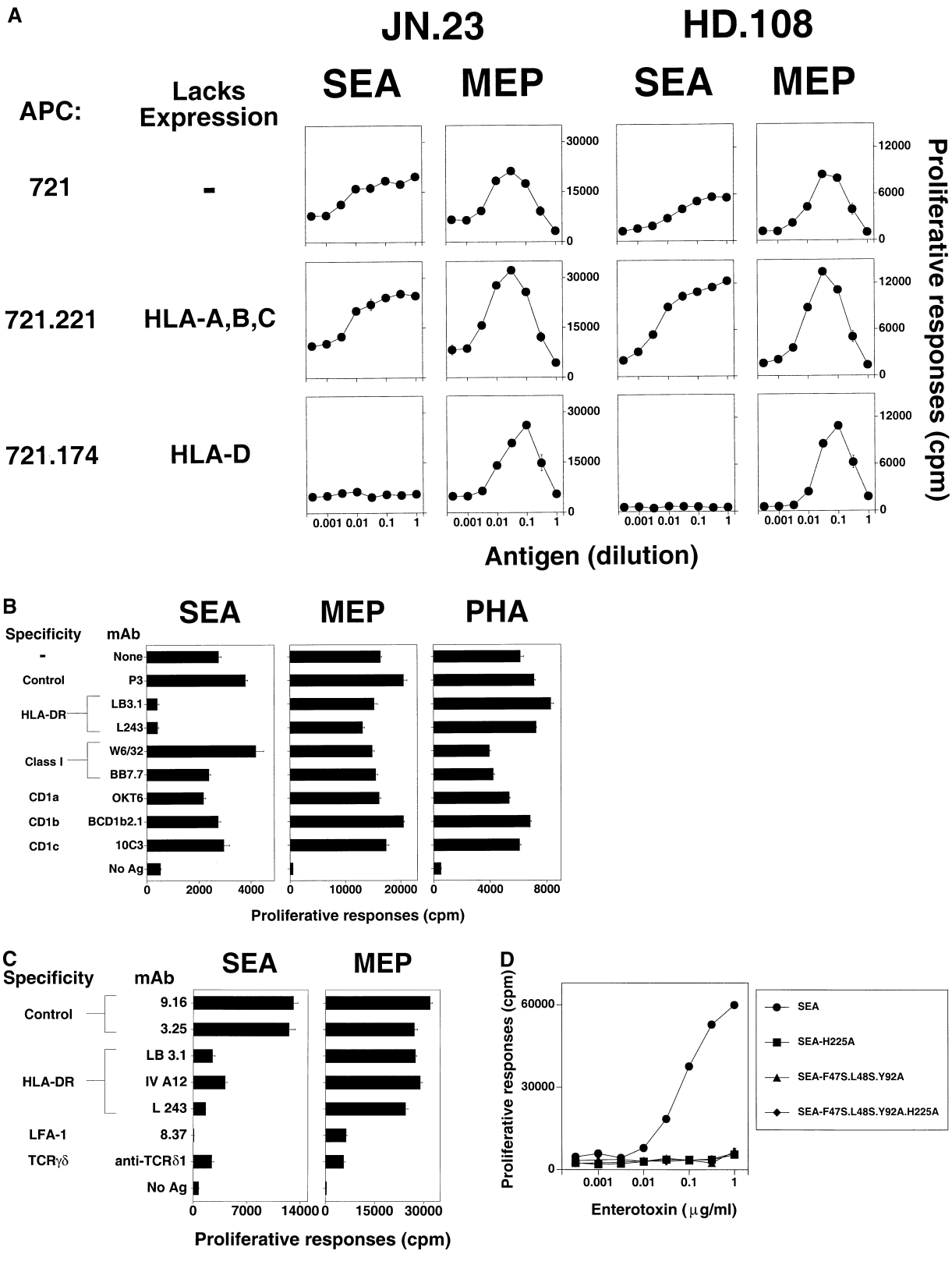


Figure 4. SEA Is Presented to γδ T Cells by MHC Class II Molecules

(A) SEA recognition by γδ T cells requires MHC class II expression, whereas prenyl pyrophosphate recognition does not. The γδ T cell clones, JN.23 (left) and HD.108 (right), were cultured with EBV-transformed B cells in the presence of one-half log dilutions of SEA (1 μg/ml) or MEP (1/100 concentration) with rIL-2 as in Figure 1A. EBV-transformed B cells used were the parent EBV line, 721, the mutant EBV line, 721.221 (lacking HLA-A, -B, and -C), and the mutant EBV line, 721.174 (lacking HLA-DR, -DQ, -DP, TAP1/TAP2, and DMA/DMB).

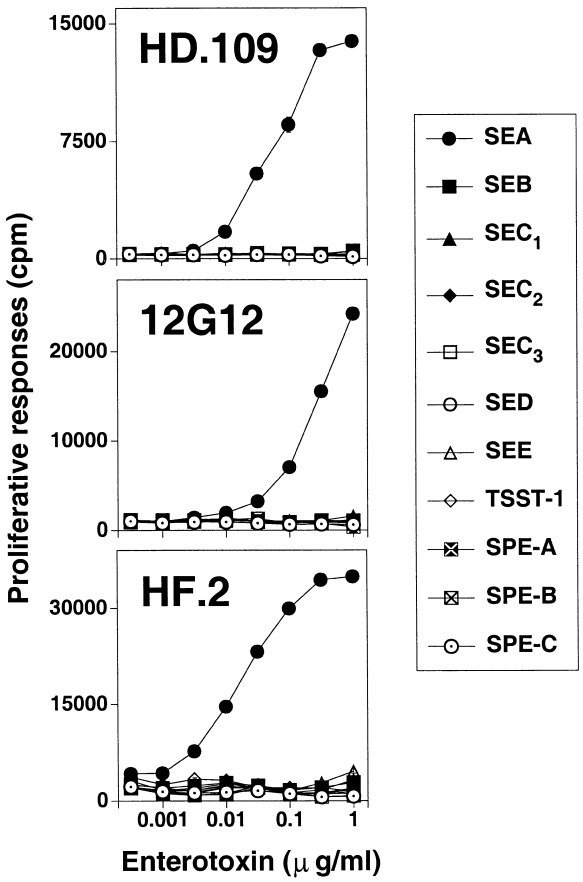


Figure 5. Vγ2$^+$ T Cell Recognition Is Specific for SEA

Vγ2$^+$ T cell clones, HD.109 (top), 12G12 (middle), and HF.2 (bottom), were cultured with SEA, SEB, SEC1, SEC2, SEC3, SED, SEE, TSST-1, SPE-A, SPE-B, and SPE-C in the presence of CP.EBV B cells with rIL-2 as in Figure 1A. Enterotoxins were added at serial one-half log dilutions starting at 1 μg/ml.

recognition by γδ T cells is similar to superantigen recognition by αβ T cells but is distinct from nonpeptide prenyl pyrophosphate and alkylamine antigen recognition. In contrast to nonpeptide antigen recognition, superantigen recognition by γδ T cells requires MHC class II molecules and the presence of antigen-presenting cells (Figures 1 and 4). Moreover, although recognition of both prenyl pyrophosphate and alkylamine antigens and superantigens is mediated by the γδ TCR, nonpeptide antigen recognition requires expression of both Vγ2 and Vδ2 domains whereas superantigen recognition required expression of the Vγ2 domain and either Vδ1 or Vδ2 domains (Figure 1A; data not shown).

The Vγ2 domain is homologous to the Vβ domain as evidenced by the ability of a VβCβ chain to pair with the VδCδ chain in one tumor cell line (Hochstenbach and Brenner, 1989), by the ability of Vα domains to productively rearrange to Cδ domains, and by the ability of Vδ1 domains to rearrange to Cα domains (Miossec et al., 1990). The Vβ domain has been demonstrated to be the site of interaction between αβ TCRs and superantigens. Consistent with this fact, the recognition site on SEA for the Vγ2 domain is localized to the region between the β-grasp and the larger β-barrel motif where the recognition site for the Vβ domain is localized (Hudson et al., 1993; Mollick et al., 1993; Swaminathan et al., 1995). Although this region is highly homologous between SEA and SEE (85% overall amino acid sequence identity), Vγ2$^+$T cells can distinguish between these superantigens. Using SEA-SEE chimeras and SEA point mutants, we have identified residues in the amino-terminal region (aa 20–27) and in the region around residue 63 as critical for Vγ2$^+$T cell recognition of SEA (Figure 7A, boxed areas). Similar regions of the SEC superantigen interact with the Vβ domain in the crystal structure of the mVβ8.2-SEC2 complex (Figure 7A , white letters in the SEC sequence) (Fields et al., 1996) suggesting that the recognition of SEA by Vγ2$^+$T cells is similar to the recognition of SEC by mVβ8.2$^+$T cells. Mutation of the amino acids around residue 63 affects αβ T cell recognition of SEB (Kappler et al., 1992) and SEA (Mahana et al., 1995). Moreover, sequence differences between SEA and SEE that are localized to the amino-terminal TCR binding region (aa 20–27) are important in SEE-specific αβ T cell discrimination between SEA and SEE, again supporting a similar model of SEA binding to Vβ domains (Hudson et al., 1993; Mollick et al., 1993; Lamphear et al., 1996).

We attempted to measure the binding affinity between SEA and the Vγ2 chain using both surface plasmon resonance with soluble monomeric and pentameric Vγ2Vδ2 TCRs and equilibrium sedimentation with a second soluble monomeric Vγ2Vδ2 TCR. In both cases, no binding was seen, probably reflecting a binding affinity between SEA and the Vγ2 chain that was below that measurable by present techniques (data not shown). We have had similar difficulties measuring the binding between human Vβ22 and SEA. Although this Vβ is strongly reactive with SEA in T cell stimulation assays, we could not detect specific binding, presumably because the affinity is also too low (unpublished data).

(B) Monoclonal antibodies specific for HLA-DR molecules block SEA recognition but not nonpeptide antigen recognition. DG.EBV B cells were used to present SEA (3.16 μg/ml), MEP (1/1000 concentration), or PHA (1/16000 concentration) to the γδ T cell clone, DG.SF68, without rIL-2 as in Figure 1A. mAbs were added as purified proteins (25 μg/ml) or as ascites fluid (1/200 concentration).

(C) Monoclonal antibodies specific for HLA-DR molecules block SEA recognition (left panel) but not nonpeptide MEP antigen recognition (right panel) by the CD4$^+$ Vγ2Vδ2$^+$ JN.23 T cell clone. EBV-transformed B cells were glutaraldehyde fixed and used to present SEA (0.1 μg/ml) or MEP (1/10000 concentration) without rIL-2 as in Figure 1A. Monoclonal antibodies were added as purified proteins (25 μg/ml) or as overgrown culture supernatants (1/4) for 30 min prior to addition of antigen. Note that antibodies to LFA-1 and TCRγδ blocked both SEA- and MEP-induced proliferation whereas antibodies to HLA-DR blocked only SEA-induced proliferation.

(D) Both the high- and the low-affinity binding sites for MHC class II on SEA are required for SEA recognition by γδ T cells. Mutant SEA toxins with point mutations disrupting the high-affinity binding site (SEA-H225A), the low-affinity binding site (SEA-F47S.L48S.Y92A), or both sites (SEA-F47S.L48S.Y92A.H225A) were added at serial one-half log dilutions starting at 1 μg/ml with the HF.2 T cell clone without rIL-2 and with DG.EBV cells as in Figure 1A.

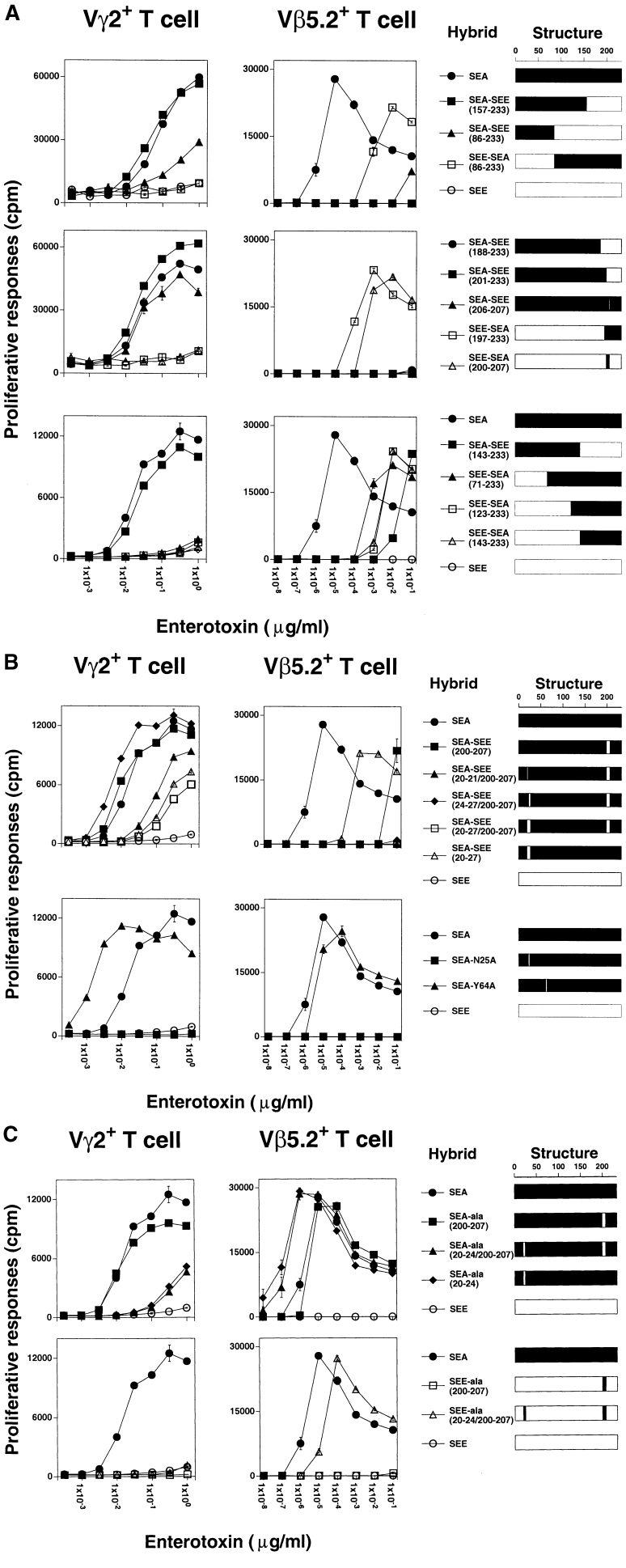


Figure 6. Amino-Terminal Residues 20–27 and 63–64 in SEA Determine Recognition by Vγ2+ TCR

Recombinant SEA/SEE chimeric and mutant SEA proteins were used to map the residues in SEA required for responsiveness to SEA by γδ and αβ T cells. The enterotoxins were added to cultures with DG.EBV B cells as APCs with either the Vγ2+ T cell clone, HF.2, or the Vβ5.2+ αβ T cell clone, Hy.198. SEA and SEE mutants (Hudson et al., 1993; Lamphear et al., 1996; Mollick et al., 1993) were added at serial one-half or one log dilutions starting at 1 μg/ml for γδ T cells and 0.1 μg/ml for αβ T cells. Cultures were as in Figure 1, except rIL-2 was omitted. Schematic representation of the chimeric molecules is shown on the right with shaded areas representing SEA and clear areas representing SEE ([A] and [B]). For alanine mutants, nonconserved residues between SEA and SEE were replaced by alanines in the area indicated (C).

(A) Residues within amino-terminal region 20–27 affect SEA recognition by Vγ2+ T cells whereas carboxy-terminal residues 206–207 in SEA affect recognition by Vβ5.2+ T cells.

(B) Residues within amino-terminal region 20–27 affect SEA recognition by Vγ2+ T cells whereas carboxy-terminal residues 206–207 in SEA affect recognition by Vβ5.2+ T cells. Note that mutation of the conserved arginine25 to alanine (N25A) completely abolished the biological activity of SEA for Vγ2+ as well as Vβ5.2+ T cells.

(C) Alanine substitutions of nonconserved residues within region 20–27 affect SEA recognition by Vγ2+ TCR. Nonconserved residues between SEA and SEE were replaced by alanine residues.

Similarly, despite proliferative responses by Vβ8.2 T cells to low concentrations of SEB, the affinity of mouse Vβ8.2 for SEB was only 144 micromolar, which was barely measurable (Malchiodi et al., 1995). This difficulty in demonstrating TCR binding to enterotoxins may reflect the fact that enterotoxin recognition requires both binding to MHC class II and to TCR. SEA may be able to compensate for low-affinity binding to TCR because its dual binding sites for MHC class II molecules results in high-avidity binding to MHC class II.

Model of Vγ2 Binding to SEA

Although γδ and αβ T cells bind to similar regions of SEA, recognition of SEA by γδ T cells is dependent on an amino- terminal region of SEA (aa 20–27), whereas recognition of SEA by SEA-specific αβ T cell is dependent on both amino- and carboxy-terminal regions of the enterotoxin molecule (Figures 6A and 6B). Residues in the carboxy-terminal region of SEA may contact Vγ2 but are not necessary for Vγ2$^+$T cells to distinguish SEA from SEE.

SEC contacts the CDR2, and to a lesser extent, the CDR1, framework 3, and HV4 regions of the mVβ 8.2 domain (contact residues [Fields et al., 1996] are in white in Figure 7B). The analogous regions of human Vγ2 (hVγ2) exhibit few sequence similarities with other SEA-specific human Vβ domains although these Vβ domains are somewhat more homologous to each other (Figure 7B). We have modeled the structure of the Vγ2-SEA complex based on a model of Vγ2 and the crystal structures of SEA and the mVβ8.2-SEC complex (Figures 7C and 7D). The Vγ2-SEA model predicts binding between the CDR1, CDR2, and HV4 regions of Vγ2 and the cleft region between the β-grasp and β-barrel motifs of SEA. A closeup view of the proposed contact regions reveals that TCR CDR2 residues aa 55–58 are predicted to make extensive contacts with SEA residues aa 21–28, and TCR HV4 residues aa 72–75 make contact with SEA residues aa 63–64. Additional contacts are predicted between TCR CDR1 aa 27–28 and SEA residues aa 102–103. Although the conserved Y205 residue of SEA is predicted to contact residues in the CDR2 loop, the polymorphic residues (G200, P206, and D207) in the carboxy-terminal region (aa 200–207) are not predicted to contact the Vγ2 chain consistent with our functional results.

Differences between Superantigen and Nonpeptide Prenyl Pyrophosphate Antigen Recognition by Vγ2$^+$T Cells

In addition to recognizing SEA, most Vγ2$^+$T cells also recognize nonpeptide prenyl pyrophosphate and alkylamine antigens. Although prenyl pyrophosphate antigens were proposed to be superantigens for γδ T cells (Pfeffer et al., 1992), we now show that the two classes of antigens are quite distinct by directly comparing γδ T cell recognition of the classical superantigen, SEA, and of the nonpeptide prenyl pyrophosphate analog, MEP. Prenyl pyrophosphate antigens are presented to Vγ2Vδ2$^+$T cells by an extracellular pathway that requires cell-cell contact but does not require known antigen-presenting molecules or professional APCs (Morita et al., 1995). In contrast, the superantigen, SEA, is presented to γδ T cells by MHC class II molecules expressed on professional APCs (Figures 1 and 4).

Although different in many respects, both superantigen recognition by αβ T cells and nonpeptide antigen recognition by γδ T cells are relatively independent of TCR diversity introduced into the CDR3 region by the recombination process (Marrack and Kappler, 1990; Panchamoorthy et al., 1991). Thus, both superantigens and nonpeptide antigens activate T cells primarily based on the germline-encoded V domain(s) used in their TCR. However, whereas SEA recognition requires only the Vγ2 domain paired with either Vδ1 or Vδ2 (Figure 1; data not shown; Loh et al., 1994), prenyl pyrophosphate antigen recognition requires both the Vγ2 and Vδ2 domains (Davodeau et al., 1993; Tanaka et al., 1994). Prenyl pyrophosphate-reactive Vγ2Vδ2$^+$T cells have fairly homogeneous junctional lengths of their TCR with rather limited diversity in the Vγ2 junction and a strong bias for the use of the Jγ1.2 junctional region (De Libero et al., 1991; Davodeau et al., 1993). Importantly, by mutating the CDR3 region of the Vγ2 chain, we have now shown that minor changes in this CDR3 region greatly affect the recognition of prenyl pyrophosphates (Bukowski et al., 1998). Thus, unlike superantigen recognition by αβ T cells, residues located within the junctional region of the Vγ2Vδ2 TCR are important for prenyl pyrophosphate antigen recognition.

Functional and Evolutionary Significance of SEA Recognition by Vγ2$^+$ T Cells

The biological significance of γδ T cell responses to SEA is uncertain. The relatively high concentrations of enterotoxin required to stimulate γδ T cells to proliferate would likely limit their systemic response to SEA produced by *Staphylococcus aureus* infection. However, the primary route of exposure to SEA is generally through the GI tract. γδ T cells, including Vγ2Vδ2$^+$ T cells, are enriched in the gut mucosa and may encounter higher concentrations of SEA in the gut than in the blood. Moreover, a higher sensitivity to SEA was noted for cytokine release where half maximal IL-2 release was at 10 ng/ml and APC fixation was not required (data not shown). Similarly, γδ T cells can kill unfixed targets in the presence of SEA (Morita et al., 1995). During various infections, Vγ2Vδ2$^+$ T cells can proliferate to very high levels of up to 30%–60% of total circulating blood T cells (Ho et al., 1990; Hara et al., 1992; Scalise et al., 1992; Sumida et al., 1992; Balbi et al., 1993; Bertotto et al., 1993; Perera et al., 1994; Ueta et al., 1994; Caldwell et al., 1995). Higher numbers of Vγ2Vδ2$^+$ T cells (>10%–15%) are found in people from developing nations (Esin et al., 1996) and in infants (Parker et al., 1990). Activation of Vγ2Vδ2$^+$ T cells by the aminobisphosphonate, pamidronate (used to inhibit bone resorption in cancer patients), can acutely cause high fevers and flu-like symptoms (Kunzmann et al., 1999), so Vγ2Vδ2$^+$ T cells can be important sources of cytokines and may help control multiple myeloma tumors (Kunzmann et al., 2000). However, as no study of γδ T cells in SEA enterotoxin food poisoning has been done, the role of γδ T cells in enterotoxin food poisoning remains unclear.

Vγ and Vβ domains diverged early in vertebrate phylogeny given that Vγ, Vδ, Vα, and Vβ T cell receptor gene

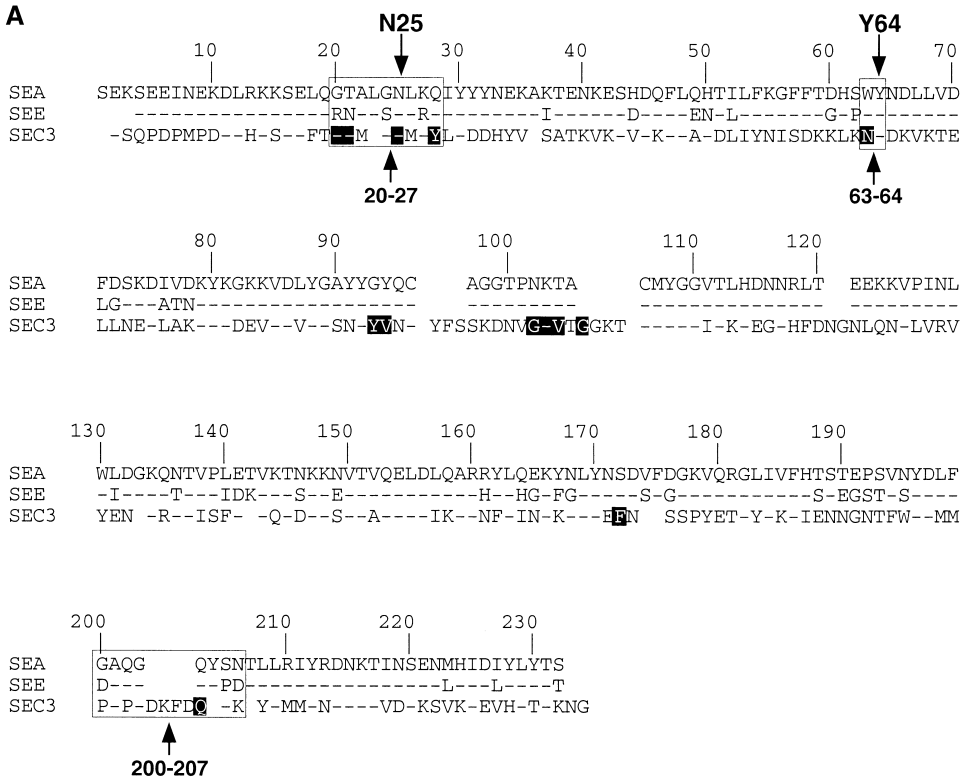


Figure 7. Model of the Vγ2-SEA Complex

(A) Comparison of the amino acid sequences of SEA and SEE. The amino-terminal region is of primary importance in Vγ2$^+$ T cell recognition (residues 20–27 and 63–64 are boxed). Changes in these regions greatly diminish, abolish, or enhance SEA recognition by Vγ2$^+$ T cells. The carboxy-terminal region of 206–207 that is structurally adjacent to the amino-terminal region 20–27 plays an important role in determining specific recognition of SEA or SEE by αβ T cells but is not important in SEA recognition by Vγ2$^+$ T cells.

(B) Sequence alignment of SEA-specific Vγ2 and Vβ chains and the SEC3-reactive mVβ8.2 chain. Sequences within and flanking CDR1, CDR2, and HV4 regions are compared between the human Vγ2 and Vβ chains. Identities are indicated with a dash. Spaces have been introduced to maximize alignment. Sequences from the SEC3-reactive mVβ8.2 chain are included for comparison. SEC3-contacting residues on mVβ8.2 chain and predicted SEA-contacting residues on hVγ2 are highlighted.

segments are found in cartilaginous fish that diverged from mammals ~450 million years ago (Rast et al., 1997). Despite this early divergence, Vγ and Vβ domains retain sufficiently similar structure such that the Vγ2 domain can interact with an enterotoxin that predominantly recognizes Vβ domains. Similar cross-reactivity may occur with SED stimulation of B cells expressing V_H4 domains (Domiati-Saad et al., 1996).

In conclusion, the determination of critical residues in SEA represents the characterization of a superantigen binding site for a γδ TCR. Furthermore, γδ T cell recognition of the SEA superantigen demonstrates that γδ T cells can recognize three classes of antigens; superantigens, alkylamines, and prenyl pyrophosphates. The finding that one superantigen activates γδ T cells suggests that there may be other superantigens primarily specific for γδ T cells. Recognition of SEA by the major subset of γδ T cells suggests that γδ T cells may contribute significantly to diseases involving this superantigen.

Experimental Procedures

Enterotoxins and Antigens

Recombinant SEA, SEE, mutant SEA, and mutant SEE enterotoxins were prepared as described (Hudson et al., 1993, 1995; Mollick et al., 1993; Tiedemann et al., 1995; Lamphear et al., 1996). In brief, enterotoxins were expressed as recombinant proteins in *E. coli* and purified by GST affinity chromatography, affinity purification, and gel filtration or red dye A affinity purification and gel filtration. A number of the enterotoxins were tested for LPS contamination and found to be negative. Nomenclature for the recombinant toxins is as described (Mollick et al., 1993; Lamphear et al., 1996). Highly purified staphylococcal enterotoxin A, B, C1, C2, C3, D, E, and TSST-1, and streptococcal enterotoxin SPE-A, SPE-B, and SPE-C, were obtained from Toxin Technologies (Sarasota, FL). Monoethyl phosphate/monoethyl pyrophosphate (MEP) was synthesized as described (Tanaka et al., 1994). Isopentenyl pyrophosphate was obtained from Sigma (St. Louis, MO). Phytohemagglutinin-P (PHA) was obtained from Difco (Detroit, MI).

Derivation and Culture of T Cell Clones

T cell lines and clones were maintained by periodic restimulation with PHA as previously described (Morita et al., 1994). The derivation of the $CD4^-8^-$ and $CD8^+$ γδ T cell clones and the weakly cytotoxic $CD4^+$ γδ T cell clones, HF.2, and JN.23, have been described (Morita et al., 1991; Spits et al., 1991; Tanaka et al., 1994; Spada et al., 2000). SP-F3 is a $CD4^+$ αβ T cell clone that recognizes tetanus toxoid C fragment (residues 947–961) (Roncarolo et al., 1988; data not shown) and responds to an unknown staphylococcal enterotoxin that contaminates some preparations of SEA from ToxTech. The HD and LG αβ T cell clones (Morita et al., 1991) and the Hy (Vβ5.2) and Mo (Vβ8) clones (Bieganowska et al., 1997) have been described. Note that all human T cell clones express MHC class II on their surfaces.

Proliferation Assays

T cell proliferation assays were performed as described (Morita et al., 1994). Assays were performed in RPMI 1640 with 8% fetal calf serum in the complete absence of human serum. Human serum was excluded from all assays to prevent possible antibody-dependent cellular cytotoxicity reported with some γδ T cells (Rust et al., 1993a). γδ T cells were generally used 4–16 weeks after restimulation to reduce background proliferation to fixed APCs. T cells were plated in triplicate in flat-bottom 96-well plates at $5–10 \times 10^4$ T cells per well with $5–10 \times 10^4$ mitomycin C-treated (unfixed) or glutaraldehyde-treated (fixed) EBV-transformed lymphoblastoid B cells. Because the γδ T cell response to nonpeptide antigens and superantigens are not MHC restricted (Kabelitz et al., 1990), allogeneic cells were suitable APC. For most experiments, B cells from the DG.EBV line (A2, 29; B7, 44, w4, w6; DR3, 7; DQw2, w4; DRw52,w53) or CP.EBV line (A3, 11; B7, w35; Cw4; DR2, DQw1) were used. Antigens or mitogens were generally added to the cultures at serial one-half log dilutions starting at 1 μg/ml for SEA, a 1/100 concentration of a partially purified MEP preparation for MEP, and a 1/100 concentration of a Difco PHA-P preparation for PHA. The cultures were pulsed with 1 μCi of ^{3}H-thymidine (2 Ci/mmol) on day 1 and harvested 16–18 hr later. In some cases, low levels of rIL-2 (0.03 nM) were added to the culture media. Mean proliferation and SEM of triplicate cultures are shown. EBV-transformed B cells were used instead of PBMC since they do not produce IL-2, eliminating the possibility of nonspecific proliferation of γδ T cells induced by IL-2 secreted by αβ T cells in the PBMC in response to enterotoxin stimulation.

Fixation and Hydroxylamine Treatment of APCs

For glutaraldehyde fixation, APCs in PBS were reacted with 0.05% glutaraldehyde (EM grade, Sigma, MO) for 15 s at room temperature followed by exposure to 0.2 M L-lysine (in H_2O at pH 7.4) for 2 min and then washed three times with PBS. Maximal SEA responses were noted when APC were fixed with glutaraldehyde concentrations of 0.05%–0.158% (data not shown); higher concentrations of glutaraldehyde decreased proliferation. For hydroxylamine treatment of glutaraldehyde-fixed APCs, APCs were fixed with glutaraldehyde as above, treated with hydroxylamine for 1.5 hr at room temperature in PBS, and washed three times with PBS. For 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (ECDI) (Pierce, Rockford, IL) fixation, APCs in PBS were reacted with freshly prepared 75 mM ECDI in 0.9% NaCl for 1 hr on ice and then washed three times with serum-free RPMI 1640.

Glutaraldehyde fixation of APC has been reported to enhance T cell reactivity to peptides due to the formation of polymeric aldehydes on the APC cell surface that form covalent bonds with lysine residues on T cell surface receptors through Schiff base formation (Rhodes, 1989, 1996; Rhodes et al., 1995). Consistent with these reports, αβ T cell responses to SEA were enhanced (3- to 5-fold shift in the dose response curve for clones Hy.65 and HD.47) when SEA was presented by glutaraldehyde fixed APCs (data not shown). A similar mechanism was responsible for SEA presentation to γδ T cells since treatment of glutaraldehyde-fixed APCs with hydroxylamine (a compound that blocks Schiff base formation) abolished the JN.23 response to SEA but only minimally inhibited the response to MEP (data not shown). Further supporting this mechanism, APCs fixed with ECDI, an agent that does not increase Schiff base formation, did not present SEA to the JN.23 γδ T cell clone (data not shown). Exposure of unfixed APC's to SEA also did not stimulate the expression of inhibitory cytokines or inhibitory cell-surface molecules (data not shown). Finally, there was no increase in SEA binding to fixed APCs (data not shown; Saito et al., 1991).

Monoclonal Antibody Blocking Studies

Monoclonal antibodies to MHC class I, MHC class II, and CD1 were: W6/32 (anti-pan class I), BB7.7 (anti-class I), LB3.1 (anti-HLA-DR), L243 (anti-HLA-DR), IVA12 (anti-HLA-DR), OKT6 (anti-CD1a), BCD1b2.1 (anti-CD1b), and 10C3 (anti-CD1c). mAbs also used in-

(C) Hypothetical model for the Vγ2-SEA complex. The modeled structure of the Vγ2 domain and the crystal structure of the SEA molecule (Schad et al., 1995) were positioned by superimposing each on the crystal structure of the mVβ 8.2-SEC2 complex. SEA was colored blue with the higher affinity Zn^{+2} MHC class II binding site colored magenta and the lower affinity MHC class II binding site colored yellow. This lower affinity site is analogous to the SEB MHC class II binding site. The Vγ2 domain was colored gray with the CDR1 (red), CDR2 (yellow), CDR3 (purple), and HV4 (green) regions colored.

(D) Closeup of proposed contacts between SEA and CDR1, CDR2, and HV4 regions of the Vγ2 domain. View is in a similar orientation to (C). SEA is colored cyan and CDR loops colored as in (C).

cluded 8.37 (anti-LFA-1), anti-TCRδ1 (anti-Cδ), and P3 (control IgG1). Prior to T cell addition, mAbs were added as either dialyzed ascites fluid (1/200 dilution), overgrown culture supernatant (1/4 dilution), or purified antibody (12.5 μg/ml) to proliferation assays with SEA, MEP, or PHA. All mAbs were shown to either block control T cell responses or to stain on flow cytometry analysis.

Modeling of the Vγ2-SEA Complex

A model of the TCR Vγ2 domain was built using the homology modeling technique. The Brookhaven PDB database was searched by AMPsrch (Collin, 1993) using the Smith-Waterman dynamic algorithm (Smith and Waterman, 1981) to select known structures that were related to the Vγ2 domain. The variable domains of the antibody heavy chains of 8FAB (F. A. Saul et al., unpublished data) and 1FVC (Eigenbrot et al., 1993) were selected due to their high resolution (1.8 Å and 2.2 Å, respectively) and sequence homology to the Vγ2 domain. A multiple sequence alignment was generated using the coordinates of 8FAB and 1FVC and modified according to the sequence and structural homology with the QUANTA 4.1 package (Molecular Simulations Inc., Waltham, MA). The main chain conformations of known structures with the highest homologies were then copied using the QUANTA 4.1 Protein Design Facility to build the backbone of the Vγ2 structure. After the initial backbone was regularized, a series of fragment database searches were performed to determine the conformation of loops, including the CDR regions. Fragments were selected if they had low values for the least-squares-fit and connected well with the surrounding residues with fewer close contacts than other fragments. The N terminus and C terminus were built to give them extensive conformation and were folded using the secondary structure prediction. The main chain conformation was regularized to optimize the geometry of the molecular structure without taking into account nonbonded interactions. The side chain conformation was checked using side chain spinning and the Karplus rotamer library by the QUANTA4.1 program. Energy minimization of the Vγ2 domain were then done by 200 cycles of steepest descent minimization followed by 1000 cycles of adopted-basis Newton-Raphson minimization using the CHARMe (polygen) force field.

The coordinates of the SEA crystal structure (Schad et al., 1995) were kindly provided by Dr. Svensson. The disordered loop, aa 59–63, in the crystal structure was modeled based on a theoretical model of SEA (Swaminathan et al., 1995) and the SEA crystal structure (Schad et al., 1995), and regularized as stated above. The model of the Vγ2-SEA complex was built by the least squares superposition of the modeled structure of the Vγ2 domain and the loop-modeled structure of SEA onto their respective structures in the crystal structure of the mVβ8.2-SEC2 complex (Fields et al., 1996). The figures were generated by the Molscript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994) programs.

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